

Examination on *Candida* spp. in refractory periapical granulomas

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Abstract

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Aim To examine the occurrence of *Candida* spp. in refractory periapical granulomas.

Methodology One hundred and three surgically removed periapical granulomas were subjected to molecular analysis for the occurrence of *Candida albicans*. DNA was extracted from the samples using a modified phenol/chloroform/isoamylalcohol method and was subjected to polymerase chain reaction (PCR) with OPA-03 and repetitive sequence (GACA)₄ primers. The PCR products were separated in agarose gel electrophoresis, stained with ethidium bromide, visualized using UV light and the sequences were analysed. Samples indicating possible occurrence of *Candida* were further investigated by histological and immunohistological methods. Periodic acid–Schiff staining (PAS) was used to detect yeast cells and hyphae, and specific monoclonal antibodies to recognize high molecular mass mannoproteins present

in the *C. albicans* cell wall. DNA extraction was controlled by running PCR using β -actin primers (a housekeeping gene). *C. albicans* CCUG19915, *C. tropicalis* ATCC750, *C. krusei* ATCC6258, *C. guilliermondii* ATCC6260 and *C. glabrata* CCUG32725 served as positive controls in PCR. A tissue preparation of chronic atrophic candidosis in oral buccal mucosa served as a positive control for histological and immunohistological examinations.

Results Polymerase chain reaction with β -actin primers indicated successful DNA extraction in 68 out of 103 samples. The majority of the samples (50) were negative whereas 18 of the samples showed PCR products indicating possible occurrence of *Candida* spp. PAS-staining and immunohistological examination of these samples were, however, negative. Further analysis of the PCR products revealed sequences not typical for *Candida* spp.

Conclusions *Candida* spp. do not seem to occur in periapical granuloma.

Keywords: *Candida albicans*, periapical granuloma, root-canal infection, yeasts.

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Introduction

In chronic apical periodontitis, inflammatory tissue develops in the area of bone resorption as a response to

intracanal microbial infection (Soames & Southam 1993). The periapical area usually remains free of bacteria, but bacteria are regularly encountered in the periapical area in acute apical periodontitis, periapical actinomycoses, osteomyelitis and other extraradicular infections (Nair 1998). However, recent studies applying sensitive DNA hybridization techniques indicate that extraradicular infections may occur more commonly and play a considerable role in the pathogenesis of apical periodontitis resistant to nonsurgical therapy (Gatti *et al.* 2000, Sunde *et al.* 2000b,c).

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Candida albicans is an adaptive oral yeast that can occasionally be isolated from the root canal in cases of persistent apical periodontitis both in pure culture and together with bacteria (Waltimo *et al.* 1997). *C. albicans* possesses a variety of virulence factors indicating its capability to survive also in the periapical area. It is a dimorphic fungus and can transform from ovoid yeast cells to elongated hyphal forms (De Hoog & Guarro 1995). Although hyphal formation is not a prerequisite for pathogenicity of *C. albicans*, biopsies of candidal infections often reveal hyphal adherence on and penetration through epithelial tissues, indicating increased pathogenicity in comparison to ovoid yeast forms (Sweet 1997) that can enhance its survival in the periapical area. One of the key virulence determinants of *Candida* species is their ability to produce secreted aspartyl proteases which digest a variety of host proteins (Hube & Naglik 2002). In addition to these major virulence factors, *C. albicans* has a tendency for phenotypic alteration affecting, for example, protease activity and contributing to environmental adaptation (Slutsky *et al.* 1985, White & Agabian 1995). This ability may assist in colonization and survival of the yeasts, and it may also lead to genetic selection of adaptive strains (Sweet 1997).

Owing to the occurrence of *C. albicans* in persistent root-canal infections and the number of virulence factors that contribute to the virulence and invasiveness, it was hypothesized that *C. albicans* may survive and infect the periradicular tissues. Therefore, the aim of this study was to examine the occurrence of *C. albicans* in periapical granulomas removed surgically in cases resistant to nonsurgical endodontic therapy.

Materials and methods

The occurrence of oral yeasts in 103 periapical granulomas was investigated using three different methods: DNA–DNA hybridization, Periodic acid–Schiff (PAS)-staining and immunohistological staining. The samples were obtained from patients suffering from persistent apical periodontitis not responding favourably to nonsurgical endodontic therapy. The nonsurgical therapy consisted of the chemo-mechanical instrumentation with 0.5–2% NaOCl, usually aiming to prepare the apical region of the canal to size 40 or bigger, and with intracanal medication with Ca(OH)₂ at least once in 2 weeks or more. The periapical inflammatory lesions were surgically removed by oral surgeons at the department of Oral and Maxillofacial Surgery, University of Oulu, Oulu, Finland between the years 1995 and 2000. The tissues were fixed in paraformaldehyde, embedded in paraffin and

then processed for histopathological analysis in a normal fashion. Samples diagnosed as periapical granuloma were selected for the study and were used with the patient's informed consent.

DNA extraction from paraffin sections

The paraffin-embedded samples were sliced into 25-µm-thick sections. The first three to five sections were discarded to eliminate possible surface contamination of the block before the collection of the samples. Then, using a new sterile blade for each sample 7–10 sections were collected for the DNA analysis. The sections were incubated in xylose for 2 × 30 min to remove paraffin followed by ethanol rinsing (100%, 30 min). The samples were then re-suspended in 220 µL of TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 8.0) and lysed twice by addition of proteinase K (final concentration, 500 µg mL⁻¹) and SDS (1% (w/v) final concentration), and incubated at 50 °C for 16 h. The resulting supernatant obtained by centrifugation at 12 000 *g* for 1 min was then extracted with phenol/chloroform and chloroform/isoamyl alcohol. DNA was precipitated by addition of NH₄OAc (100 µL, 10 M) and ethanol (900 µL, 100%) and incubated at –20 °C for 24 h. DNA was separated by centrifugation at 12 000 *g* for 30 min at 4 °C. The supernatant was removed, 70% alcohol was added and the suspension was centrifuged at 12 000 *g* for 15 min at 4 °C. The supernatant was removed and the pellet was dissolved in 50 µL of sterile water, and the samples were stored at 4 °C.

Primers

Primers for *Candida* detection were OPA-03 (5'-AGT-CAGCCAC-3') and repetitive sequence (GACA)₄ according to Hannula *et al.* (1997). In order to control the DNA extraction, all samples were examined first by using β-actin primers (Coombs *et al.* 1999).

Polymerase chain reaction

The polymerase chain reaction (PCR) was performed as follows: 10 µL of cell lysate diluted 1 : 50 in sterile water was used as DNA template in a 30-µL PCR reaction volume consisting of 200 µM dNTPs, 1 mM of both primers, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂ and 3 U DyNAzyme 2 polymerase (Finnzymes, Helsinki Finland). The thermocycling program consisted of initial denaturation at 94 °C for 5 min and 47 cycles were run under the following conditions: template denaturation

at 94 °C for 1 min, primer annealing at 32 °C for 1 min and extension at 72 °C for 2 min (Hannula *et al.* 1997). The PCR products were separated on 1% TBE agarose gels, stained with ethidium bromide (1 µg mL⁻¹), and visualized and photographed on an ultraviolet transilluminator.

Positive control samples with yeast DNA and a negative control consisting of all reaction components except the template were included to each PCR run. *C. albicans* CCUG19915, *C. tropicalis* ATCC750, *C. krusei* ATCC6258, *C. guilliermondii* ATCC6260 and *C. glabrata* CCUG32725 served as positive controls. The stock isolates were stored at -20 °C and cultivated on commercially available yeast culture plates (Sabouraud Dextrose Agar, Tammer-Tutkan Maljat Oy, Tampere, Finland) at 37 °C for 48 h. DNA was extracted from single colonies as described above.

Histochemical stainings

The immunohistological staining was done with the monoclonal primary antibody 3H8 raised against a zymolase-solubilized preparation from blastoconidia cell walls of *C. albicans* ATCC26555 (kindly provided by the Société de Recherche et de Realizations Biotechnologiques, Paris, France) (Marcilla *et al.* 1999). The staining was performed on 4 µm formalin-fixed, paraffin-embedded sections using Vectastain[®] Elite ABC Kit 6101 (Vector Laboratories, Burlingame, CA, USA) according to the instructions with slight modifications. After deparaffinization and pepsin incubation the sections were incubated in 0.3% H₂O₂ in methanol to quench the endogenous peroxidase activity. Nonspecific binding was blocked with 2% normal blocking serum (Vectastain[®] Elite ABC Kit). The sections were incubated with the primary antibody (diluted 1 : 500 in 1% BSA:PBS) for 30 min at 37 °C and kept overnight at 4 °C in a humid chamber. Control staining was performed by omitting the primary antibody. After three washes the sections were incubated for 30 min at 37 °C with biotinylated anti-mouse IgG secondary antibody (1 : 200) and with Vectastain[®] Elite ABC reagent. Peroxidase binding sites were revealed with 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO, USA) diluted in *N,N*-dimethylformamide (Merck, Darmstadt, Germany), 0.05 M acetic acid and 0.05 M sodium acetate, pH 5.0. The sections were counterstained with Mayer's haematoxylin and mounted in glycergel (DAKO Corporation, Carpinteria, CA, USA). Periodic acid–Shiff staining was completed according to routine histological procedures. Oral buccal mucosa sections with previously diagnosed *C. albicans* infection

were used as positive control samples for PAS and immunohistochemical stainings.

Sequence analysis

The PCR-amplified DNA products were sequenced in an automated ABI Prism 377 DNA Sequencer with dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). The PCR primers were used also as sequencing primers. The sequence analysis was carried out using BLAST program (Altschul *et al.* 1997).

Results

The analysis protocol and the results obtained in each step are demonstrated in Fig. 1. Polymerase chain reaction with β-actin primers indicated successful DNA extraction in 68 out of 103 samples. The majority of the samples (50) were negative whereas 18 samples showed PCR products indicating possible occurrence of *Candida* spp. All the negative PCR controls remained without reaction products whereas positive controls revealed characteristic PCR profiles for each *Candida* strain. However, PAS-staining and immunohistological examination of the PCR-positive samples were all negative. All positive control sections in histological and immunohistological examination revealed clear *C. albicans* infection. Analysis of the PCR products revealed sequences not typical for *Candida* spp.

Discussion

The occurrence of *C. albicans* in periapical granulomas resistant to nonsurgical endodontic therapy was examined using three different methods: DNA–DNA

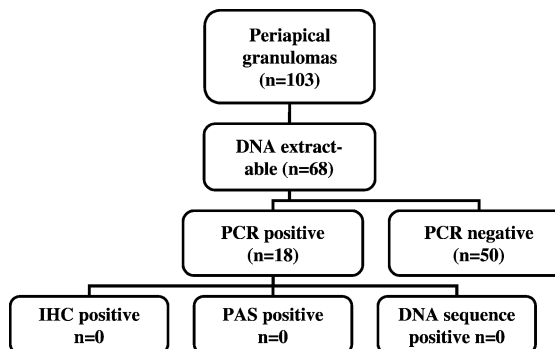


Figure 1 The protocol of the different steps of the analysis along with the number of samples with the respective finding.

hybridization, PAS-staining and immunohistochemical staining with monoclonal antibody. These methods were chosen owing to their different nature and sensitivity. Furthermore, the sequences of DNA–DNA hybridization products were analysed to explain the apparent conflicts between the findings obtained with the different methods.

DNA extraction was successful in only 68 out of 103 samples (66%), possibly because of the extraction of DNA from paraffin-embedded tissue samples instead of freshly extracted samples. Although fixation preserves tissue architecture and proteins, extraction of nucleic acids may be difficult, yielding degraded DNA only. Coombs *et al.* (1999) tested nine different extraction methods, the success ranging from 0 to 83.7%. Therefore, the rate of successful DNA extraction from the paraffin-embedded samples in the present study is acceptable. In spite of the expected methodological difficulties, a retrospective approach was chosen in order to gain a sufficient amount of samples representing periapical granulomas, paying regard to the relative infrequent occurrence of yeasts (7–21%) in root-canal infections (Waltimo *et al.* 1997, Baumgartner *et al.* 2000).

Eighteen of the samples showed PCR products indicating possible occurrence of *Candida* spp. However, neither examination with monoclonal antibodies nor PAS-staining revealed *Candida* in these samples. Further analysis of the PCR products revealed sequences not typical for *Candida* spp., indicating that the primers used were not specific enough. However, these particular primers have been successfully used by Hannula *et al.* (1997, 1999) for genotypic characterization of oral *Candida* spp. Obviously, the specificity of the primers is sufficient for examining pure yeast cultures but not for tissue samples such as investigated in the present study. This emphasizes the importance of the primer selection, as well as the use of alternative methods to confirm the findings, when the presence of microbes in periapical lesions is examined with DNA-based techniques. Furthermore, the encountered nonspecific DNA multiplication indicates the high sensitivity of this method that therefore requires strict controls to avoid false positive results.

Neither molecular nor morphological evidence for the occurrence of *Candida* spp. in periapical granuloma was found in the present study. This is in contradiction with a recent report by Sunde *et al.* (2002a), in which *Candida* was cultivated from periapical lesions not responding favourably to nonsurgical therapy, even if the number of lesions with *Candida* was small. Although *C. albicans* has a number of virulence factors required for tissue penetration, our results support the traditional

concept that host defence factors limit infection within the root-canal system (Nair 1997, 1998). However, owing to the dynamic balance between root-canal infection and host defence, small amounts of microorganisms may be detected from periapical lesion using sensitive techniques (Sunde *et al.* 2000b,c). Furthermore, some bacteria such as *Actinomyces* spp. and *Arachnia propionica* are particularly virulent, and may therefore occasionally cause extraradicular infections hosting large numbers of microorganisms (Happonen 1986, Sjögren *et al.* 1988).

The present findings suggest that the association of *C. albicans* with persistent root-canal infections (Waltimo *et al.* 1997, Baumgartner *et al.* 2000) cannot be explained with periapical occurrence of yeasts. Rather it is likely that this association is owing to resistance to treatment procedures of nonsurgical endodontic therapy. These include resistance of microorganism to calcium hydroxide and increased resistance even to common irrigants owing to biofilm formation and penetration into dentinal tubules (Sen *et al.* 1995, 1999, Waltimo *et al.* 1999, 2000).

Conclusions

The use of advanced detection methods to detect microorganisms in periapical lesions may improve our understanding, particularly concerning the causative and regulating factors of persistent periapical lesions. However, great care should be exercised when using DNA-detection-based techniques because of their sensitivity and possibility of false positive findings. It would be beneficial to confirm the results obtained with DNA techniques using other methods. *C. albicans* has been associated with root-canal infections resistant to nonsurgical therapy, and paying regard to its virulence factors, it is a potent pathogen to infect periapical lesions. However, the present study provided no evidence to support this hypothesis.

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